Chapter 1: Introduction and outline of the thesis

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1.1. Tissue engineering of skeletal muscle

Tissue engineering aims to develop tissue substitutes that resemble native tissue by providing instruction to the cells to restore and mimic the lost tissue [1]. Preferably, the tissue substitutes are composed of the patient's cells meaning that the immune response is minimal, and the newly implanted tissue is integrated and recognized by the body as its own. The constructed tissue uses different materials, synthetic or natural, as scaffolds to guide the cell growth and organization. Material properties such as wettability, charge, topography, stiffness, and geometry can dictate the cells' fate [2] (Fig. 1).

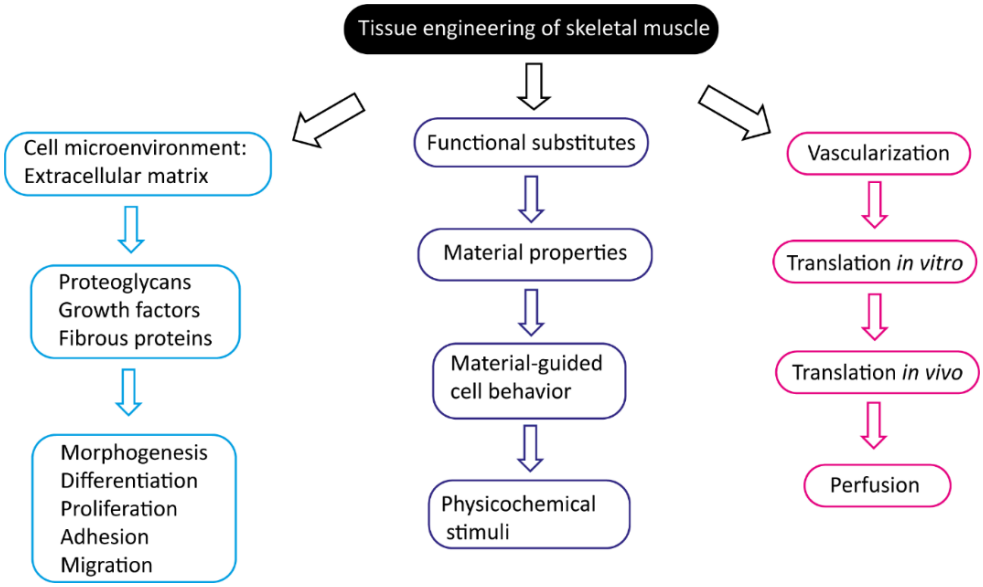


Figure 1: Diagram of tissue engineering requirements and considerations needed to create an appropriate engineered substitute. Tissue engineering needs to consider the native extracellular matrix (ECM) in order to reproduce the *in vivo* environment in an *in vitro* setting. In order to do that, the functional substitutes require to comply with a series of mechanical and chemical properties that allow cells communication and survival, besides the exchange of nutrients provided by the vascularization and guarantees the tissue integration onto the body.

The cellular microenvironment, the extracellular matrix (ECM), is a delicate network of proteins where the cells of the body reside. The ECM affects the processes of morphogenesis, differentiation, proliferation, adhesion, and migration [2–4]. Each organ and tissue have their own extracellular matrix characteristics and these need to be considered accordingly when engineering and designing the tissue engineered substitute [5].

Skeletal muscle

Skeletal muscle is a highly organized tissue which supports locomotion, respiration, and posture [6]. It has renewal capacity thanks to quiescent stem cells called satellite cells which reside on the myofibers' sarcolemma [6]. Once activated, these cells are called myoblasts, and they fuse to form myotubes. Later, myotubes continue to fuse and mature, becoming myofibers. Skeletal muscle is structured as three layers of connective tissue: the endomysium, which surrounds the myofibers; the perimysium, which is located around groups of myofibers called bundles; and epimysium, which is the layer around the whole muscle [7, 8]. The skeletal muscle ECM contains the basal lamina and the fibrillar reticular lamina. The basal lamina is located in the surroundings of the myotubes and is composed of non-fibrillar collagen, mainly collagen type IV [9], and non-collagenous glycoproteins such as laminin and proteoglycans [6, 10]. The fibrillar reticular lamina corresponds to the interstitial connective tissue [4, 8, 11], which is mainly composed of collagen type I [7]. The ECM in the muscle allows the transmission of force, and cell repair and maintenance, by affecting the reservoir of satellite cells [8].

Vascularization in tissue engineering

The production of large-sized tissue-engineered substitutes is still one of the obstacles to overcome in the field. To date, successful tissue-engineered substitutes are tissues that are thin or are avascular such as skin and cartilage [12, 13]. In the human body, tissues are supplied with nutrients and oxygen through a network of capillaries which are at a maximum distance of 200 µm from each other, the so-called diffusion limit [13]. Thus, tissues greater than 150 - 200 µm require pre-formed vasculature in order to properly integrate into the host to avoid necrosis [12, 14, 15].

The extracellular microenvironment of macrovascular cells (diameter larger than 100 µm) consists of organized collagen fibers named accordingly to their microstructure: intima (disperse fibers), media (fiber bundles at 30°), and adventitia (axially aligned fibers) layers [16]. The tunica intima is composed of endothelial cells (ECs), the media of vascular smooth muscle cells (VSMCs) and the adventitia of a diverse population of myofibroblasts [17]. The extracellular microenvironment of microvascular cells (diameter less than 100 µm) is composed of mural cells called pericytes which share the same basement membrane as ECs [17, 18].

Vascular remodeling depends on the process of angiogenesis driven by chemotaxis, defined as a chemical gradient, primarily of VEGF and Ang-2 [14, 17, 19]. During Angiogenesis, ECs migrate from existing vasculature by developing tip cells which guide the vessel sprout. At the same time, proliferative ECs, stalk cells, support the tip-cell migration to encourage vessel branching [14, 17]. Angiogenesis is also dependent on the cellular micro-environment and cellular adhesion to the ECM [11, 17]. For example, ECM-cell interaction with fibronectin and interstitial collagens stimulates EC tubular formation [20]. Thus, EC

migration processes are also affected by gradients of ligands (hapotaxis), stiffness (durotaxis) [16, 19], and as has been more recently noted, topography (topotaxis) [21].

Requirements for tissue engineering of skeletal muscle

An important aspect of tissue engineering of skeletal muscle is to consider the different processes, biochemical and physicochemical, and the geometry/morphology of the native tissue as accurately as possible. By knowing the characteristics of the muscle microenvironment, the design and implementation of solutions for the replacement of impaired or lost muscle can be deciphered.

Cells in their natural microenvironment are constantly subjected to different biochemical and physical stimuli [22]. Different strategies have been used to try to implement these microenvironment characteristics into the design of materials to recreate natural interactions between cells, e.g. by creating biomaterials, such as hydrogels with different stiffness, porosity, or architectures [23]. In addition, stem cells from different origins have various preferences for material properties [23]. Thus, the ideal biomaterial should mimic the target tissue and direct the cells of interest to act as in their native ECM.

One of the most important aspects to reproduce from native skeletal muscle tissue is aligned organization. Myofibers have an anisotropic nature which is supported by the collagen fibers which are parallel to the muscle and stop cells from over-elongation and contraction [9]. Thus, for tissue engineering of skeletal muscle it is vital to reproduce this aligned orientation of its ECM onto the substrate chosen for the engineered substitute.

Understanding the material-guided cell behavior

Understanding the cellular response to its environment helps biomaterial design. The cellular response to the ECM occurs through cellular sensing. This is possible due to surface membrane receptors. These receptors are called integrins, when the interaction is between the cell and the extracellular matrix (ECM), and cadherins, when the receptor is responsible for mediating cell-cell interactions. Once cells sense a change in their environment, they go through a modification of their biochemistry and gene expression [24].

Cell microenvironment affects cell fate by controlling the process of signaling for activation or quiescent states. This signaling affects both intrinsic (transcription factors) and extrinsic mechanisms (growth factors, extracellular matrix, cell-cell contacts) [25, 26]. Cell microenvironment differs in every tissue of our body and each specific niche is not yet fully understood [26]. Mechanisms guiding cell behavior are influenced by the material properties and the biomechanical factors exhibited by them such as rigidity/stiffness, mechanical loading, cell adhesion, cell spreading, shear stress, cyclic strain, and architectural properties that include topography, geometry (2D or 3D), and dimensionality [2, 3] (Fig. 2.).

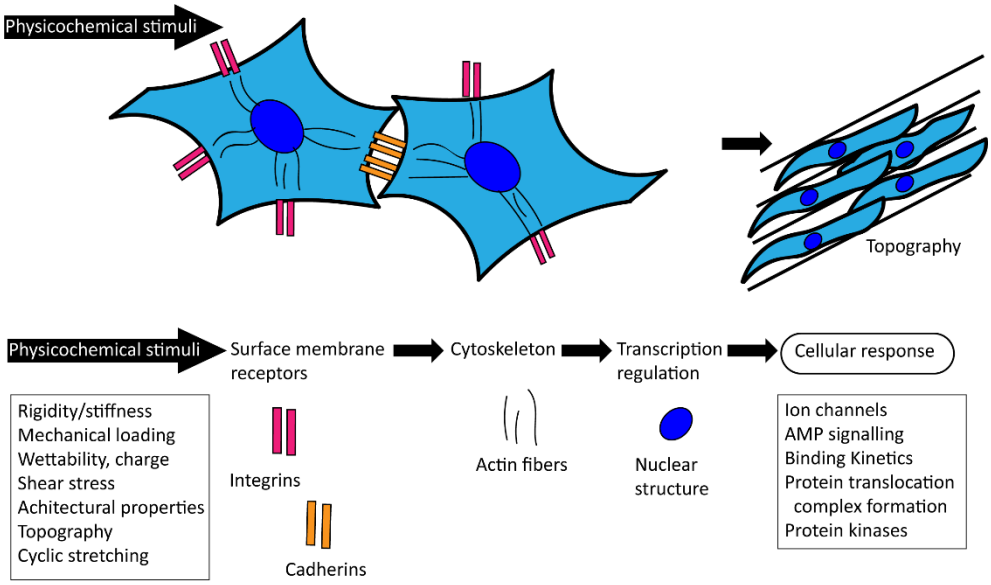


Figure 2: Cell sensing: How cells respond to physicochemical stimuli. The Physicochemical stimuli activate the surface membrane receptors. Subsequently, focal adhesions, formed by clustering of integrins, are linked to the actin cytoskeleton and the nucleus. Once in motion, they activate a variety of cellular responses that affect the cell fate.

Current strategies for tissue engineering of skeletal muscle

Scaffold design aims to reproduce the natural cell response obtained in the native tissue in an *in vitro* setting. Different strategies have been used for tissue engineering of skeletal muscle although a functional vascularized skeletal muscle has not yet been developed. This is mainly due to a lack of vascularization and upscaling approaches. For implanting a large-scale muscle, pre-vascularization is required. Depending on the material properties and fabrication methods, the most common techniques implemented to research the alignment of the skeletal muscle *in vitro* have been 3D strain alignment, contact guidance and cell sheet technologies.

3D strain alignment

Bioartificial muscle models (BAMs) [27–29] and myobundles [30–32] are the most well-known 3D systems in tissue engineering of skeletal muscle (Fig. 3). They are based on placing the myoblasts on a fibrin or fibrin/Matrigel system anchored at the edges by Velcro that facilitates the introduction of tension. As a result, aligned myotubes are created in a biodegradable and biocompatible matrix that can be implanted in animal models. In

addition, these systems allow the placement of electrodes on the edges and implement an electric stimulation to produce a cyclic strain recreating exercise function. However, these systems lack understanding of the extracellular matrix environment created and the implications of the contents of different proteins and cell types: e.g. ECs and fibroblasts in the mechanical behavior of the formed bundle. These systems have been vascularized successfully *in situ* in animal models [31, 33] but sizes do not exceed 1.5 mm in diameter and 2 cm in length [28, 33] and lack of pre-vascularization inhibits their use for larger muscle tissue. In addition, in some cases the resulting muscle tissue has lacked cross-striation [29] which means maturation of the muscle is still required. Therefore, bioartificial muscle models and myobundles application have been limited to drug testing systems.

Systems that use contact guidance

Contact guidance refers to the capacity of cells to sense their substrate, reorganize their cytoskeleton according to the shape of the surface, and follow its directionality [34–36]. There are different substrates to induce the cell's cytoskeleton reorganization. The substrates vary in sizes from molecular to micron-sized features. The main difference between these substrates depends on the fabrication methods used for their creation, which results in distinct geometries and topographies. Fabrication methods are widely review elsewhere [37–42]. Nano pits can be created by electron beam lithography [43], and nanopillars by photolithography [44]. These examples are part of the geometries and topographies used for contact guidance, but here the focus will be on those that are aligned because this is the natural geometry that mimics the ECM of the native muscle.

One of the most common geometries that causes alignment is micropattern technology. These patterns are created using surface chemistry to generate stripes of e.g. fibronectin [45, 46] or cell adhesive peptide sequences [47, 48] (Fig. 3). Studies with micropatterns ranging from 20 µm to 200 µm have found that murine origin cells and human cells differ in their response of differentiation efficiency [45] and cell spreading area [48]. In addition, myoblasts' pattern architecture preference is linked to the surface chemistry e.g. laminin formed larger myotubes [45]. Thus, most of the literature available relies on the C2C12 murine origin cell line that cannot be translated into the human model. Additionally, these systems base their myoblast contact guidance only on surface chemistry which can affect the differentiation properties of the myoblasts [45].

Microgrooves (surface topography) are usually created by soft lithography [39]. Microgrooves usually have sharp-edged grooves with patterns ranging from 800 nm width, 800 nm groove and 600 nm ridge [49] or 100 µm width, 50 µm groove and 50 µm ridge [50]. Sinusoidal architectures have been also used, albeit to a lesser extent, with features ranging from wavelengths of (λ) 280 nm to 2 µm and amplitudes (A) of 8 nm to 450 nm [51]. Moreover, different materials, PLGA and hydrogels (GelMA), or PDMS have been used for the differentiation and alignment of myoblasts.

Aligned nano/micro-fibrils are fabricated by using electrospinning using synthetic materials such as poly(ε-caprolactone) (PCL) [52, 53], or hybrid fiber matrices such as PCL/collagen [54], PLGA-collagen type I and graphene oxide fibers [55], or natural 85% fibrinogen and 15% alginate [56]. Nano fibers of approximately 300 nm in diameter to micro bundles of 30 mm long and 800-1100 µm in diameter have been studied for the culture of skeletal muscle cells [54–56] but it still is unknown which parameters and materials produce the best results since different materials and cell types have been used for this purpose.

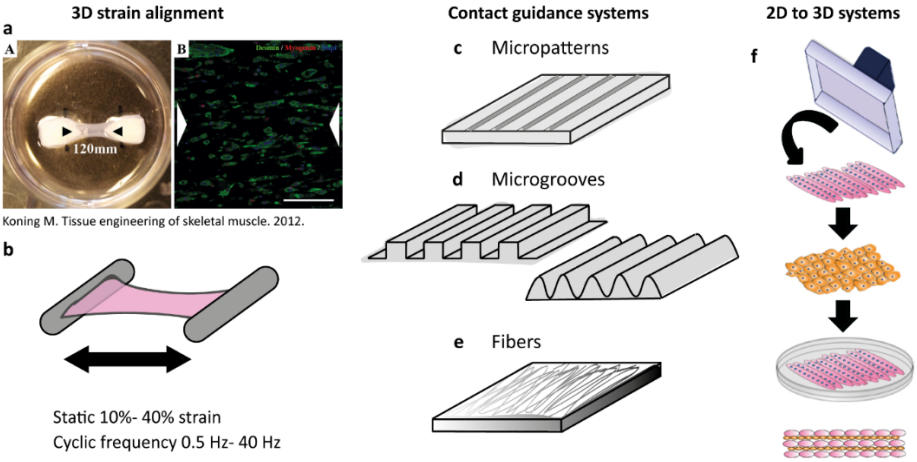
2D to 3D systems: Cell sheet technology

Cell sheet engineering allows conversion of a 2D culture into a 3D [57] culture system in order to improve the thickness of the engineered muscle (Fig 3). However, this technique is still in its infancy. It has been possible to manipulate the cell layer stacking with the use of thermo responsive materials such as NIPAAm [58–62] and techniques such as gelatin plungers [63]. The best strategy for tissue engineering of skeletal muscle has yet to be identified.

Other techniques that are capable of creating 3D systems include 3D bioimprinting [64]. Although these systems seem a promising tool in tissue engineering of skeletal muscle, questions of how to upscale the process by developing accessible techniques and materials remain. Besides, understanding the natural processes of the body helps to engineer platforms that make use of the natural stimulation routes to facilitate regenerative processes for tissue engineering or drug-screening platforms.

Different techniques have been used but no functional substrate has been approved for human use nor has a protocol been developed to ensure the creation of a functional muscle. Understanding of the mechanisms guiding cell behavior and the different cues dictating it can benefit the design of biomaterials. Thus, one first approach is to recognize the different cellular responses when changing one variable at a time from their cellular niche. Topography is one of these variables.

(Next page) **Figure 3**: **a.** Co-culture of HUVECs and myoblast on collagen type I-Matrigel gel using Velcro anchoring points as a strain alignment system. **b.** General representation of different strain systems that use fibrin, Matrigel, or collagen type I as a base to create a 3D alignment system with static or cyclic strain [33, 56] with the most observe values used in the field. **c.** Representation of micropatterns, resulting from surface chemistry treatments to obtain layers of proteins on stripes that guide cell alignment. **d.** Representation of systems of microgrooves, usually fabricated by soft lithography, contained architectures with grooves, ridges and depths. However, sinusoidal features are also produced. **e.** Aligned fibers which resulted from electrospinning using special collector designs (e.g. magnetic field-assisted collector [52]) for control the deposition of the fibers. **f.** Representation of the cell sheet technology in order to upscale a 2D cell culture to a 3D system.



Topographic systems

Topography influences cell contact guidance from the nanoscale to the microscale in 2D systems by directing the cells to follow the topography directionality [65, 66]. Clusters of integrins form focal adhesions which are responsible for cell adhesion and attachment to different surfaces and materials [65]. Besides, focal adhesions are connected to the actin cytoskeleton which simultaneously communicates with the nucleus and therefore activates a cascade of cell responses and alters cellular behavior [66] (Fig. 2). Topography has been proven to affect the directionality of the actin fibers [67] and therefore the cell directionality. In addition, topography has also aligned myoblasts [49–51, 59, 61, 68–70]. Alignment of myoblasts allows the formation of aligned myotubes that mimic the ones found *in vivo*. However, most of the research has been done with the murine origin-cell line C2C12, which does not accurately represent the response of human myoblasts [71]. Additionally, it remains unclear which topographical features are best for the culture and differentiation of human myoblasts.

Sinusoidal substrates can be generated with the technique: shielded surface oxidation with air plasma [72] (Fig. 4). Briefly, films of cured PDMS are stretched and surface oxidation with air plasma is performed. A gradient was generated by using an angle mask that protects the substrate and facilitates the formation of an surface oxidation gradient [73]. In the past, we have shown that directional gradients are screening platforms to evaluate osteoblasts, bone marrow-derived mesenchymal stromal cells [67, 73], and adipose tissue-derived stromal cells' (ASCs) [74] alignment response. Therefore, directional topography as a strategy for tissue engineering of skeletal muscle seems appropriate if optimum features are identified to allow proliferation and differentiation of myoblasts and ECs by using the directional gradient technology.

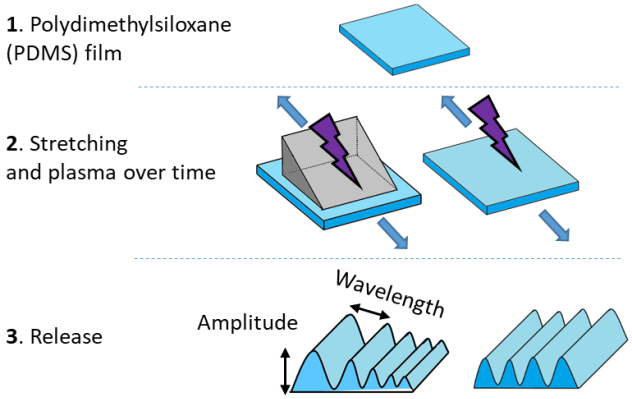


Figure 4: Surface oxidation using air-plasma. The first step is to create a PDMS film. Secondly, this is stretched, and a mask can be used to control its exposure to the air-plasma. It is exposed for a certain time, which affects its stiffness. Finally, the PDMS is released from the tension and sinusoidal patterns are created in the surface. Figure adapted from [73].

Approaches for vascularization

As previously mentioned, the main challenge for researchers attempting to achieve vascularization is to overcome the diffusion limit: where tissues larger than 150-200 µm need pre-formed vasculature to allow the exchange of nutrients and gases. Once this challenge has been overcome, large tissues could theoretically be created *in vitro* before implantation into patients. However, pre-vascularization in tissue engineering is still a work in progress.

Previous work of our group identified that human umbilical vein ECs and retinal ECs formed sprouting networks on monolayers of adipose stromal cells [75–77]. This was important for recognising the ASCs as precursors for vascularization.

Alignment of endothelial cells has been less studied than that of skeletal muscle cells [46, 78]. Electrospinning has also been used to create endothelial cell alignment [79], and co-cultures with aligned myoblasts [29] have been investigated, resulting in successful co-cultures that have the ability to last for over a week. However, the cues surrounding the vascularization response of skeletal muscle monocultures remain unknown. The same applies to the topotactic responses of ECs in different environments and niches of the human body.

1.2. Outline and aim of the thesis

Despite the advances in the development of *in vitro* skeletal muscle as a drug screening platform, no functional skeletal muscle for tissue engineering purposes has been fully developed to date. The aim of this thesis is to identify which topography is linked to the alignment, proliferation, and differentiation of human myoblasts in conjunction with endothelial cells to engineer pre-vascularized skeletal muscle tissue. Topography is a way to control the cell behavior in order to create desired responses such as morphological arrangements, which are part of the structural architecture of skeletal muscle.

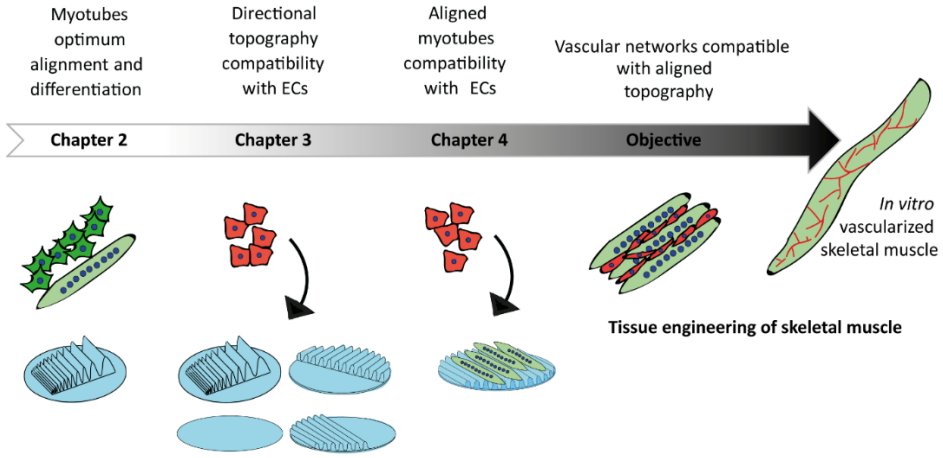


Figure 5: Outline of the thesis

Topography has been shown to guide the cellular behavior and the proliferation and differentiation of myoblasts. However, a large pool of evidence showed discrepancies in which topographical features were best suited for the purpose of aligning human myoblasts along with other factors such as the approach to vascularization, which were addressed in this chapter (**chapter 1**), the basic concepts that this thesis addresses. In **Chapter 2** the optimum alignment of human myoblasts is investigated: Directional topography gradients drive optimum alignment and differentiation of human myoblasts. We hypothesized that myoblasts have a preferred directional topography to proliferate, fuse, and mature to myotubes.

In addition, it was necessary to evaluate how our topography influences endothelial cell alignment and sprouting. In **Chapter 3** the response of endothelial cells towards topographical gradients is investigated to assess their alignment and sprouting response to different topographical features. We speculated that a variety of topographies influence sprouting network formation and alignment of endothelial cells.

In **Chapter 4** identifying the protein deposition pattern of myotubes on the directional topography is discussed as well as how this is influenced by directionality. In addition, we wanted to evaluate if the myotubes aligned in the topography were able to sustain the sprouting of endothelial cells aiming for the pre-vascularization of our system *in vitro*. Therefore, we hypothesized that myotubes instead of myoblasts should sustain sprouting.

In **Chapter 5** the findings are discussed, and conclusions drawn as to how topography and ECM can dictate the deposition and morphology of skeletal muscle and endothelial cells. Future perspectives in tissue engineering of skeletal muscle using topographical systems are also discussed.

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